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Multiple roles for FGF-3 during cranial neural development in the chicken

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SUMMARY

FGF-3 has been implicated in the development of the hindbrain and otocyst in vertebrate embryos. Since the chicken embryo offers a favourable system in which to study the development of these structures, we have isolated and characterised cDNAs for chicken *Fgf-3* and determined its pattern of expression in chick embryos from stage 3 (primitive streak) to stage 25 (early organogenesis). Within the developing cranial neural tube, *Fgf-3* exhibits dynamic spatial and temporal expression. During extension of the head process, RNA is detected in the midline of the developing neural plate. In neurulating embryos, transcripts are observed initially in rhombomeres 4 and 5 of the hindbrain and later, in rhombomere 6. During hindbrain development, expression is lost from these rhombomeres, but becomes restricted to rhombomere boundaries, providing an intracellular marker which distinguishes a population of cells within boundary regions. *Fgf-3* expression is elevated in ventral and medial boundary regions and is greatly reduced in dorsal parts. Studies of

regenerating rhombomere boundaries show that *Fgf-3* expression is induced in reforming boundaries when even-numbered rhombomere tissue is grafted next to odd, but not when like is juxtaposed to like. *Fgf-3* disappears from boundary regions just prior to the loss of the morphological boundaries suggesting a boundary-associated function.

Other sites of expression have also been identified. At early stages of development *Fgf-3* is expressed in the epiblast and mesendoderm of the primitive streak, in mesoderm lateral to the streak and in Hensen's node. In older embryos transcripts are detected in the endoderm of the pharyngeal pouches, the ectoderm of the second and third pharyngeal arches and the stomodeum. Expression was also detected in the segmental plate and in the posterior half of the three most-recently generated somites.

Key words: *Fgf-3*, embryo, hindbrain, rhombomere, rhombomere boundary

INTRODUCTION

During the development of the vertebrate neural tube, the cranial neuroepithelium becomes organised into a series of morphological segments called neuromeres. Within the developing hindbrain, these transient structures are called rhombomeres (Gräper, 1913; Adelman, 1925; Vaage, 1969). Considerable insight into the possible relevance of this hindbrain segmentation has been obtained from studies using the chicken embryo. During the initial period of segmental development, rhombomeres are polyclonal compartments of cell lineage restriction; that is cell movement is prevented between them (Fraser et al., 1990). Further evidence for the influence of segmentation is provided by the spatial origins of branchiomotor nuclei; cell bodies of the cranial motor nerves in avian embryos are located in pairs of rhombomeres (Lumsden and Keynes, 1989). In addition, segmentation of the hindbrain appears to play a crucial role in the regulation of pharyngeal arch development and patterning by the neural crest. Neural crest cells, which emigrate from the dorsal region of the hindbrain into the pharyngeal arches, pattern the skeletal elements and associated musculature which form within each arch (reviewed by Noden,

1988). Recently, the majority of neural crest cells that populate each arch have been shown to originate in different rhombomeres (r) in the avian hindbrain: cells from r1 and r2 populate the first pharyngeal arch, cells from r4 populate the second arch, and r6 and r7 cells populate the posterior arches. r3 and r5 do not produce significant amounts of neural crest (Lumsden et al., 1991; Sechrist et al., 1994) and this reduction appears to be regulated by adjacent even-numbered rhombomeres (Graham et al., 1993, 1994). Signalling from the hindbrain neuroepithelium may also influence the development of the adjacent ectoderm, in particular the development of the otic placode (see below).

Each rhombomere also has a unique molecular identity: axial limits of the expression of *hox* gene transcripts occur at boundaries between rhombomeres and *Krox-20* mRNA and protein are restricted to rhombomeres 3 and 5 (reviewed by Graham, 1992; Wilkinson, 1993; see also Prince and Lumsden, 1994 and references therein). In addition to these transcription factors, molecules associated with intercellular signalling are differentially expressed between rhombomeres, these include Cwnt-8C (Hume and Dodd, 1993) and the receptor tyrosine kinases (RTKs) *sek* and *ret* (Nieto et al., 1992; K. Robertson

and I. M., unpublished data). To date however, only one potential RTK ligand has been reported to be expressed in a segmentally restricted fashion in the hindbrain; fibroblast growth factor-3 (*Fgf-3*, formerly known as *int-2*; Wilkinson et al., 1988).

There are currently nine members of the fibroblast growth factors (FGF) family. Most are secreted constitutively, they bind to proteoglycans in extracellular matrix and on the cell surface and signal through a family of four transmembrane receptor tyrosine kinases (fibroblast growth factor receptors, FGFRs; reviewed by Mason, 1994). *Fgf-3* expression has been detected in r5 and r6 of the mouse embryo hindbrain at 9 days of development, prompting speculation that FGF-3 might play a role in the development of the otocyst which invaginates adjacent to r5 and r6 (Wilkinson et al., 1988). Indeed, there is experimental evidence which suggests that signals emanating from the otic region of the hindbrain are required for the induction and later morphogenesis of the otocyst (reviewed by Van de Water and Represa, 1991). Evidence that FGF-3 might influence otic development has been obtained from studies of both chicken and mouse embryos. Antisense oligonucleotides and FGF-3 antisera have been shown to inhibit otic pit invagination in explant cultures of embryonic chicken tissue (Represa et al., 1991). By contrast, mice homozygous for a targeted disruption of the *Fgf-3* gene have apparently normal otocyst formation but have defects in later stages of otic development albeit with incomplete penetrance (Mansour et al., 1993). It is important to note that the studies in the chicken embryo necessitated the use of reagents which were based on the available mammalian sequences, since at the time both the sequence of the chicken *Fgf-3* gene and its expression in the developing hindbrain of this species were not known.

We have now isolated and characterised cDNAs for the chicken *Fgf-3* gene and determined its pattern of expression in the chick embryo. In the developing neural tube, we have found that *Fgf-3* RNA is expressed in the midline of the cranial neural plate at pre-somitic stages, subsequently in r4, r5 and r6 during neurulating stages and finally expression is localised exclusively to rhombomere boundaries. We evaluate its potential role in otic vesicle invagination and have used the expression of *Fgf-3* RNA as a marker to study the reformation and induction of rhombomere boundaries in manipulated embryos. Other sites of *Fgf-3* expression in the chicken embryo are also described and are discussed with reference to data from other vertebrate species.

MATERIALS AND METHODS

Isolation of chicken *Fgf-3* cDNA clones

Genomic DNA was isolated from the livers of 15-day old chicken embryos as described by Costantini and Lacy (1981). Oligonucleotide primers, GCAGATCTAGAGARTGCGARTTCGTNGAR and GCAGATCTAGAIAGCACYCKIGGNAGRAA were designed against the conserved FGF-3 amino acid sequences LGYNTY and FLPRVL and included *Bgl*III and *Xba*I restriction sites for cloning purposes. These were used to amplify a 177 bp fragment of the chicken *Fgf-3* gene using the polymerase chain reaction (PCR) with *Pfu* polymerase (Promega, Madison, USA) in the manufacturer's buffer under the following cycling conditions: 94°C for 2 minutes then 40 cycles of 94°C for 2 minutes, 47°C for 3 minutes and 72°C for 3 minutes. PCR products were cloned into pGEM-4Z (Promega,

Madison, USA) by standard procedures. This fragment was sequenced and shown to encode protein sequences compatible with that expected for chicken FGF-3. This clone was used as a probe to screen a library prepared from a stage 15 chick embryo cDNA in λ ZAP (Nieto et al., 1994; kindly provided by D. Wilkinson). A number of clones were isolated and the largest were sequenced using the Sequenase system (Amersham International, Slough, UK). Sequences were assembled and analysed using the GeneWorks Programme suite (Intelligenetics Inc., Mountain View, CA, USA).

In situ hybridisation

Rhode Island Red hen's eggs were incubated to stages 3-25 (Hamburger and Hamilton, 1951) in a humidified atmosphere. Following dissection, in situ hybridisation to whole embryos was undertaken as described by Wilkinson (1992) with the following alterations. Embryos were dissected into Howard's Ringer and post-hybridisation washing was altered as follows: three washes were performed in solution 1 each for 30 minutes at 70°C. Washes in solution 2 and RNase A were sometimes omitted, proceeding immediately from solution 1 to solution 3 of the original protocol. Embryos were pre-incubated in 10% sheep or goat serum for up to 4 hours. After incubation with anti-digoxigenin, alkaline phosphatase-coupled fab fragments (Boehringer, Lewes, UK) embryos were washed 5 times for 1 hour each with TBST and incubated in TBST overnight prior to incubation with substrate. After sufficient coloured product had developed, embryos were washed in several changes of PBT prior to storage in sterile PBT or 90% glycerol, 1× PBS. Probes for in situ hybridisations were prepared from the original *Fgf-3* PCR fragment.

After in situ hybridisation, embryos were either mounted on slides in sterile glycerol (early stages), dissected and mounted (late stage hindbrains) or prepared for sectioning. Either thick (vibrotome) or thin (wax) sections were cut. For thick sections, embryos were embedded in a mixture of 0.5% gelatin, 30% egg albumin, 20% sucrose in PBS and sections were cut at 50 μ m and mounted in glycerol. For thin sections, embryos were post-fixed in 4% paraformaldehyde in PBS for 4 hours, dehydrated in 100% methanol (5 minutes), 100% propan-2-ol (10 minutes) and tetrahydronaphthalene (30 minutes). Embryos were equilibrated in 50:50 tetrahydronaphthalene:wax (Paraplast; Sherwood Medical Co., St. Louis, USA) at 58°C for 30 minutes, then in three changes of wax each for 1 hour at 58°C, and embedded in wax. Sections were cut at a thickness of 15-20 μ m, de-waxed in xylene and mounted under DPX (Merck, Poole, UK).

Removal of rhombomere boundaries by aspiration

To examine reformation of a rhombomere boundaries and associated *Fgf-3* expression, the r3/r4 boundary was removed from embryos at stage 11 (13 somites) as described by Guthrie and Lumsden (1991). The r3/r4 boundary was chosen because it is the first to show restricted *Fgf-3* expression within the boundary region. Eggs were windowed and embryos were visualised by sub-blastodermal injection of Indian ink. The boundary region was aspirated using a microelectrode attached to a mouth pipette. This manipulation was performed bilaterally in order to eliminate the possibility of cells migrating across the floor plate from the opposite side of the hindbrain. Operated eggs were sealed with Sellotape™ and incubated for between 0 and 18 hours.

Formation of new rhombomere boundaries by grafting

Eggs were windowed at stages 10-11 (10-13 somites) and prepared for use as either donor or host embryos. Donor embryos were dissected from eggs and maintained in Howard's Ringer while pieces of hindbrain were excised bilaterally using a tungsten needle; providing tissue for two host embryos. Anterodorsal sides of the transplants were marked with carmine (Sigma, Poole, UK). Host embryos were visualised in the egg using Indian ink. Hindbrain pieces were excised unilaterally; the contralateral side served as a control.

Hindbrain regions were excised such that associated boundary regions were removed but floor plate and notochord were left intact (for further details see Guthrie and Lumsden, 1991). Transplants were performed such that r3 and r5 or r3 and r4 were juxtaposed. Operated eggs were sealed and incubated to stage 15 (25 somites).

RESULTS

Isolation and characterisation of chicken *Fgf-3* cDNAs

The predicted amino acid sequences of human, mouse and *Xenopus* proteins (Moore et al., 1986; Brookes et al., 1989; Tannahill et al., 1992; Kiefer et al., 1993a) were compared and oligonucleotide primers were designed from two conserved regions that are encoded by a single exon in the mouse and human genes (Moore et al., 1986; Brookes et al., 1989). These primers were used to amplify a fragment of the chicken *Fgf-3* gene from genomic DNA and this fragment was used to screen a chicken embryo cDNA library to isolate further clones containing complete FGF-3 coding sequences. Four cDNA clones were isolated, with the largest being 4 Kb. The sequence of the predicted coding region obtained from sequencing these clones along with its derived amino acid sequence is shown in Fig. 1A. The conceptual protein sequence is 220 amino acids in length with a M_r of 25×10^3 for the unmodified polypeptide. There is a predicted signal

sequence for secretion (von Heijne, 1986) with a cleavage site between amino acids 18 and 19, in agreement with the identified cleavage site for mouse FGF-3 (Fig. 1A; Kiefer et al., 1994). Comparison with the protein sequences of human, mouse and *Xenopus* FGF-3 is shown in Fig. 1B and confirms

A

ATGCTCGTGATCTGGCTCTGCTGCTGCCCTGTGCCCCGAGCCGCGCTCCCGCCGCTACGGCCTCCCCGAGG	75
M L V I W L L L L A L L P E P R V P A A T A S P R	
-----↑	
GCCCCGCGGATGCGGGGGGCGCGCGCGCTCTACGAGCACCTCGGGGAGCGCCCCGCCGAGGAAGCTCTAC	150
A P R D A G G R G G V Y E H L G G A P R R R K L Y	
TGTGCCACCAAGTACCACCTGCAGATCCACCCGCGGCAAGATCAACGGCACCTTGAGAAAAACAGCGTCTTC	225
C A T K Y H L Q I H P G G K I N G T L E K N S V F	
-----*	
AGTATCCTTGAAATAACTGCTGTGTGATGTCGGGATCGCTATCAAGGGCTTGTCTCTGGCAGATACCTGGCC	300
S I L E I T A V D V G I V A I K G L F S G R Y L A	
ATGAACAAAGGGGAGGCTTTATGCATCAGAGAACTATAACACAGAAATGTGAGTTTGTGGAGAGGATTCATGAA	375
M N K R G R L Y A S E N Y N T E C E F V E R I H E	
CTGGGCTACAACACCTATGCATCCGCTGTGTACCGAACTGTACCCAGTGGAGCCAGCACCAAGCGCAAAGCCAGT	450
L G Y N T Y A S R L Y R T V P S G A S T K R K A S	
GCGGAGAGACTCTGGTACGTCTCAGTCAATGGGAAAGGACGACCCAGGAGGGGCTTTAAACACGCAGGACACAG	525
A E R L W Y V S V N G K G R P R R G F K T R R T Q	
AAATCTCCTCTTTCTGCCAGAGTATTGGATAGCAAAGACCATGAAATGGTCCGACTTTTCCACACAAATGTG	600
K S S L F L P R V L D S K D H E M V R L F H T N V	
AGATACCGAGAGAGCCTCCTGAAGCCTCCAGTAAGAACCAGAGGAGAAGGAGGAGGACGCTGA	663
R Y R E S L L K P P S K N Q R R R R G R .	

B

Chicken FGF-3	MLVIWLLLLL LL---P--- -----EPRV PAATAS----	33
Xenopus FGF-3	MVIIWLLLLL FISCGPQVSW AKRLEREPKY PCSRGGKLCD PRDRRDAGGR	50
Human FGF-3	MGLIWLLLLL LL----- -----EPGW PAAGPG----	32
Mouse FGF-3	MGLIWLLLLL LL----- -----EPGW PTTGPG----	32
Chicken FGF-3	GGVYEHLGGA PRRKLYCAT KYHLQHLPGG KINGILEKNS VFSILEITAV	83
Xenopus FGF-3	GGVYEHLGGA PRNRKLYCAT KYHLQHLNG KINGILEKNS VFSILEITAV	100
Human FGF-3	GGVYEHLGGA PRRKLYCAT KYHLQLPSPG RVNGELE-NS AYSILEITAV	81
Mouse FGF-3	GGVYEHLGGA PRRKLYCAT KYHLQLPSPG RVNGELE-NS AYSILEITAV	81
Chicken FGF-3	DVGIVAIKGL FSGRYLAMNK RGRLYASENY NPECEFVERI HELGYNTYAS	133
Xenopus FGF-3	DVGIVAIKGL FSGRYLAMNQ RGRLYASETY NPECEFVERI HELGYNTYAS	150
Human FGF-3	EVGIVAIKGL FSGRYLAMNK RGRLYASEHY SAECEFVERI HELGYNTYAS	131
Mouse FGF-3	EVGIVAIKGL FSGRYLAMNK RGRLYASDHY NAECEFVERI HELGYNTYAS	131
Chicken FGF-3	RLYRTVPSSA STRRKASADR LWYVSMNGKG RPRRGFKTRR TQKSSFLPR	183
Xenopus FGF-3	RLYRTVPSSA GTRRKASADR LWYVSMNGKG RPRRGFKTRR TQKSSFLPR	200
Human FGF-3	RLYRTVSSTP GARRQPSAER LWYVSMNGKG RPRRGFKTRR TQKSSFLPR	181
Mouse FGF-3	RLYRTSSSP GACRQPSAER LWYVSMNGKG RPRRGFKTRR TQKSSFLPR	181
Chicken FGF-3	VLDNRDHEMV RLFTNV-RY RESLLKPPSK NQ-RR-----	217
Xenopus FGF-3	VLDNRDHEMV RLFTNA-VY RESILKPSKP SG-RQ-----	234
Human FGF-3	VLDNRDHEMV RLQSGSLPRP PGKGVCPRRR RQ-KQSPDNL EPSHVQASR	229
Mouse FGF-3	VLDNRDHEMV RLQSSQPRP PEGESQPRR RQKQSPGDH GKMETLSTRA	231
Chicken FGF-3	-RGR-----	220
Xenopus FGF-3	-RGQ-----	237
Human FGF-3	-LGSQLEAS- ---AH	239
Mouse FGF-3	TPSTQLHTGG LAVAX	246

Fig. 1. Sequence of the predicted coding region of chicken *Fgf-3* cDNAs and comparison of the conceptual amino acid sequence with other vertebrate FGF-3 protein sequences. (A) Sequence of the protein encoding sequence of chicken *Fgf-3*. The probable signal sequence for secretion is underlined and its cleavage site is indicated by an arrow. The single predicted site for N-linked glycosylation is marked by an asterisk. Nucleotides are numbered. (B) Comparison of the predicted protein sequences of the AUG-initiated isoforms of chicken, *Xenopus*, human and mouse FGF-3. Identical amino acid residues conserved among all four FGF-3 proteins are boxed. Amino acid residues are numbered. The sequences have been deposited in the EMBL and GenBank databases; accession no. Z47555.

the identity of the chicken *Fgf-3* clone. The chicken amino acid sequence has strong homology to human and mouse sequences (67% and 64% identity respectively), but is most similar to the *Xenopus* protein (78% amino acid identity). The single N-linked glycosylation site that is conserved in FGF-3 from other vertebrate species and implicated in secretion (Fig. 1B; Dixon et al., 1989; Acland et al., 1990; Kiefer et al., 1991, 1993b) is also present in the chicken protein sequence (amino acid 66; Fig. 1A), but potential sites of O-linked glycosylation described for mouse FGF-3 (Kiefer et al., 1993b) are not conserved in the avian protein. The predicted chicken protein is smaller than its homologues in other vertebrates, lacking both the amino-terminal insertion seen in the *Xenopus* protein and the carboxy-terminal extensions of the mammalian FGF-3 proteins.

Expression in the primitive streak and somites

Expression of *Fgf-3* RNA was examined in chicken embryos between stages 3 (onset of gastrulation) and 25 (absence of morphological hindbrain boundaries). The first site of weak *Fgf-3* expression was at stage 3 in the developing primitive streak (data not shown). At stage 4, strong expression was observed in ectoderm along the length of the streak and around Hensen's node, in developing mesendoderm in the primitive groove and in mesoderm migrating away from the streak (Fig. 2A,F). In all older embryos examined, *Fgf-3* transcripts were observed in the ectoderm and mesoderm of the primitive streak (Fig. 2B-D,H and data not shown) and also in the segmental plate, the most recently formed notochord tissue and in the posterior half of the three most recently formed somites (Fig. 3I and data not shown).

Expression in the midline of the cranial neural plate

Coincident with the extension of the notochord from Hensen's node in mid-stage 4 embryos (4+; full length primitive streak), *Fgf-3* was detected in the midline of the developing neural plate just anterior to the node (Fig. 2B). At stage 5 (head process, Fig. 2C) this expression extended rostrally to the rostral limit of the neural plate and, at stage 6 (head fold), there was a reduction in the apparent transcript levels (Fig. 2D). Transverse sections of stage 5 embryos, taken anterior to Hensen's node, showed *Fgf-3* RNA in cells in the midline of the neural plate; these being the precursors of the ventral part of the cranial neural tube including the floor plate (Fig. 2E). By stage 7 (onset of somitogenesis), expression in the midline of the cranial neural plate was no longer detectable (Fig. 2H).

Expression in the developing hindbrain

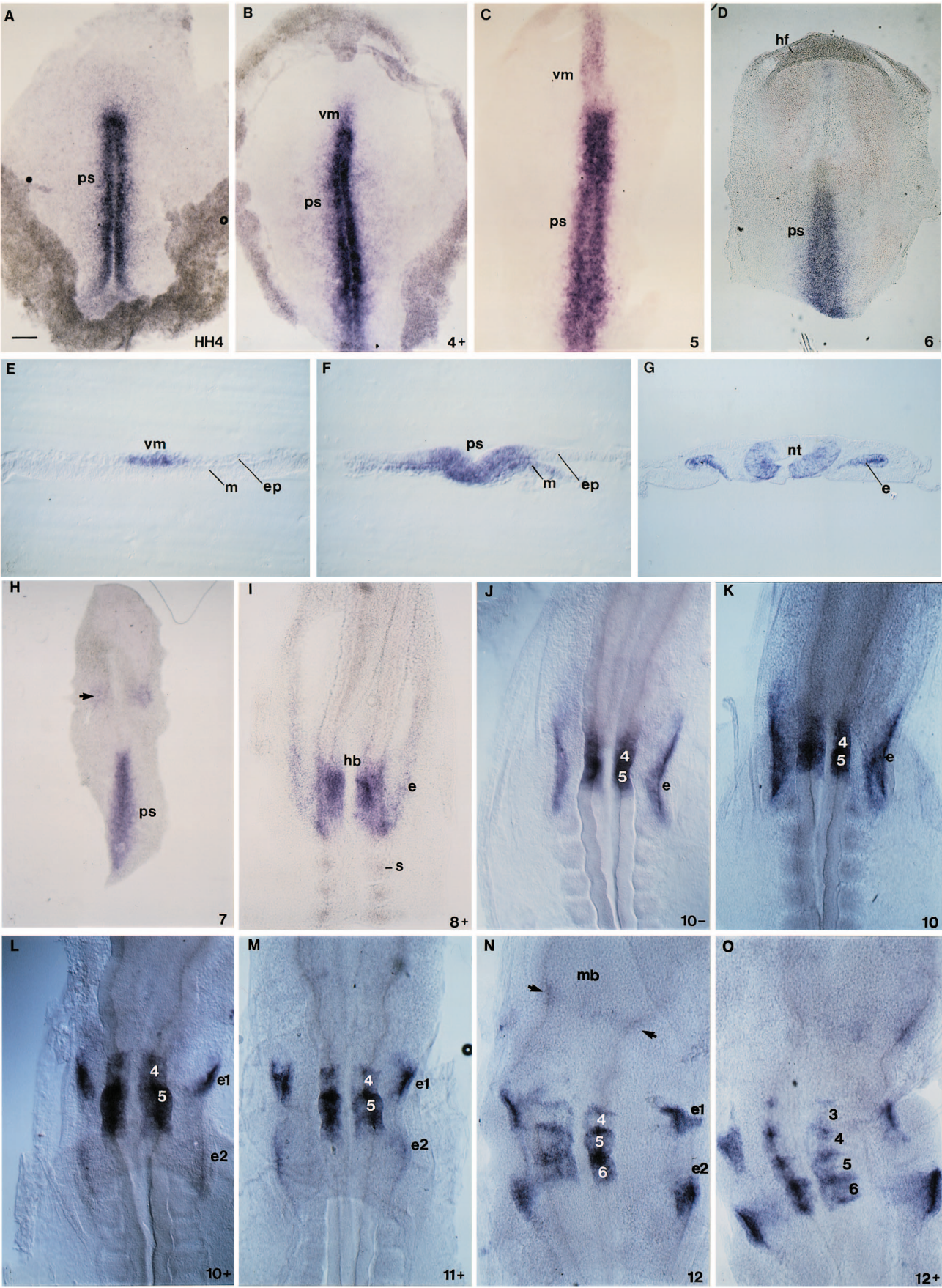
A detailed analysis of *Fgf-3* transcripts during hindbrain development revealed an unexpectedly dynamic spatial and temporal pattern of expression. Expression in this region was first observed in a patch of neuroepithelium at stage 7, coincident with the loss of detectable RNA in the midline of the cranial neural plate. Following the appearance of rhombomeres, expression was restricted to r4, r5 and later, r6 while still later, expression was associated specifically with cells in rhombomere boundaries.

At stage 7, weak *Fgf-3* transcripts were first detected in a region of the unsegmented hindbrain just anterior to the first somite and, notably, not in the midline of the neural plate in this region (Fig. 2H). Similar expression was observed until

Fig. 2. Expression of *Fgf-3* in chicken embryos between stages 4 and 12+. Developmental stages are indicated in the lower right corners.

(A) Stage 4 (appearance of groove in primitive streak) embryo: transcripts extend along the streak and around Hensen's node. (B) Stage 4+ (formation of head process): expression is maintained in the primitive streak and Hensen's node but is also detected in a region anterior to the node. (C) Stage 5 (head process): *Fgf-3* RNA is detected in the midline of the embryo; in the streak, the node and extending from the node to the rostral tip of the embryo. (D) Stage 6 (head fold): expression is maintained in the streak and the regressing node but is reduced in the cranial midline. (E,F) Transverse sections of a stage 5 embryo. (E) Section taken rostral to Hensen's node shows that transcripts are restricted to the midline of the neural plate, the prospective ventral and floorplate regions of the cranial neural tube. (F) Section taken through the primitive streak shows expression in the epiblast cells of the streak and the emergent mesendoderm cells. More laterally, expression is restricted to mesoderm derivatives. (G-O) Expression in the developing hindbrain and pharyngeal region. (G) Transverse section taken through the r4/r5 region of a stage 10 embryo. Transcripts are detected in the pharyngeal pouches and the hindbrain neuroepithelium. Expression is detected in both ventricular and medial cells but is excluded from the floorplate and most dorsal region of the hindbrain. (H-O) Dynamic spatial expression of *Fgf-3* within the developing hindbrain. (H) Stage 7 (onset of somitogenesis, 1 somite: 1s): expression is maintained in the primitive streak but is also just detectable in the developing hindbrain (arrow), just anterior to the level of the first somite. RNA is detected in this region either side of the midline but is not detected in the midline itself. (I) Stage 8+ (5s): transcripts are present in the posterior hindbrain in a domain that is anterior to the first somite and, laterally, in the endoderm. (J) Stage 10– (onset of rhombomere formation; 9s) embryo: expression is observed in the rhombomere 4 and 5 (r4, r5) territory of the hindbrain neuroepithelium and in the developing pharyngeal endoderm. (K) Stage 10 (10s): transcripts are detected throughout r4 and in the anterior of r5. (L) Stage 10+ (11s): *Fgf-3* RNA becomes reduced in r4 and expression extends throughout r5 and into the anterior of r6. Transcripts are now detected in two regions of the developing pharyngeal endoderm corresponding to the first and second pharyngeal pouches. (M) Stage 11+ (14s): expression becomes reduced in the body of rhombomere 4 but not in the r3/r4 boundary region. Transcripts are detected more caudally in r6. (N) Stage 12 (16s): reduced transcript levels are observed in the bodies of r4, r5 and r6 but levels remain elevated in rhombomere boundaries. *Fgf-3* RNA is also detected at the midbrain/hindbrain boundary (arrows). (O) Stage 12+ (17s): *Fgf-3* RNA is now detected in the r2/r3 boundary. Abbreviations: e, endoderm; e1, endoderm of the first pharyngeal pouch; e2, endoderm of the second pharyngeal pouch; ep, epiblast; hb, hindbrain; m, mesoderm; mb, midbrain; nt, neural tube; ps, primitive streak; s, somite; vm, ventral midline of neural plate. Individual rhombomeres are numbered. Scale bar: (A-C) 20 µm; (D,H), 40 µm; (I-O), 10 µm and (E-G) 5 µm.

stage 8+, although apparent transcript levels were increased (5 somites, Fig. 2I and data not shown). The morphogenesis of rhombomeres in the chicken hindbrain begins at stage 9– (6 somites) with the appearance of the r5/6 boundary and is completed at stage 12 (16 somites) with the formation of the r1/2 and r7/8 boundaries (Vaage, 1969; Lumsden, 1990). At stage 9 (7 somites) the r3/4 and r5/6 boundaries are present and *Fgf-3* expression had a rostral limit at the r3/4 boundary with expression extending caudally into r5; thus transcripts were initially restricted to r4 and r5. Similar expression was observed until stage 10– (9 somites, Fig. 2J and data not shown). However, between stages 10 and 10+ (10–11 somites),



expression began to extend progressively more caudally into the prospective r6 region. At the same time, transcript levels began to be reduced in r4 but were maintained in the r3/4 boundary region (Fig. 2K, L). Transverse sections of the r4/5 region of a stage 10 embryo showed that transcripts were expressed throughout the neuroepithelium with the exception of the floorplate and the most dorsal regions of these rhombomeres (Fig. 2G). Between stage 11– (12 somites) and stage 11+ (14 somites), apparent levels of *Fgf-3* RNA continued to drop in r4 and also began to be reduced in r5 (Fig. 2M and data not shown). At the same time, the relative transcript level in r6 increased, until at stage 12 (16 somites, Fig. 2N), there was no detectable expression in r4, weak expression in r5 and strong expression throughout r6.

Although *Fgf-3* expression was becoming reduced within rhombomeres 4 and 5 themselves, RNA was maintained at high levels in their respective rhombomere boundaries (r3/r4; r4/r5 and r5/r6). By stage 12– (15 somites, data not shown), *Fgf-3* RNA was also detected in the r2/r3 and prospective r6/r7 boundaries. Interestingly, expression became elevated in the r6/r7 boundary region before its morphological appearance at stage 12 (16 somites, Fig. 2N). Between stage 12+ (17 somites) and stage 13– (18 somites), expression in r5 and r6 was further reduced, by contrast expression in all boundaries between r2/r3 and r6/r7 was maintained at higher levels (Fig. 2O and data not shown). By stage 13 (19 somites) *Fgf-3* expression was restricted to these boundary regions and this pattern of *Fgf-3* expression was observed in embryos until stage 15 (25 somites, Figs. 3A, B). Examination of flat-mounted hindbrains showed that expression was detectable in the ventral and medial regions of the boundaries, reduced in boundary cells dorsally and excluded from the floorplate (Fig. 3C). Cells positive for *Fgf-3* were detected extending from the ventricular to pial surface of the neuroepithelium in a region having the 'fan shape' morphology that is characteristic of rhombomere boundaries (Fig. 3B; Heyman et al., 1993). Expression in cells at the ventricular surface of ventral boundary regions extends approximately 6 cell diameters in the rostrocaudal axis, again consistent with the morphological dimensions of apparent boundary regions (Heyman et al., 1993), and suggesting that most ventral boundary cells express *Fgf-3* RNA.

At stage 19 (approximately 38 somites, data not shown) *Fgf-3* expression was reduced in the r6/r7 boundary, but by stage 22 (Fig. 3C) weak expression was also observed in the r1/2 boundary. In later stages, expression in all rhombomere boundaries was progressively reduced such that by stage 24 (Fig. 2D) no expression was detected in boundary regions. This occurred just prior to the loss of morphological boundaries at stage 25 (Vaage, 1969; Lumsden, 1990). *Fgf-3* transcripts were never observed in the r7/8 boundary. The spatial and temporal pattern of *Fgf-3* expression and its correlation with the appearance of morphological rhombomeres is summarised in Fig. 4.

Regulation of *Fgf-3* during regeneration and formation of rhombomere boundaries

Guthrie and Lumsden (1991) have described the reformation of rhombomere boundaries following the removal of an existing boundary by aspiration. We examined the regulation of *Fgf-3* expression in such regenerating boundary regions. We studied the reformation of the r3/4 boundary in stage 12 embryos, since at that stage expression in r4 itself was no

longer detectable. The r3/4 boundary was removed on both sides of the floorplate to exclude the possibility of repopulation by cells migrating from the contralateral boundary region as previously described (Guthrie and Lumsden, 1991). After removal of the r3/r4 boundary, *Fgf-3* expression was examined in embryos immediately after the boundary was removed (0 hours) and following incubation for up to 18 hours, when the boundary has completely reformed (Guthrie and Lumsden, 1991). Examination of embryos immediately after boundary removal showed that no *Fgf-3*-positive cells remained associated with the posterior of r3 or the anterior of r4. However, by 8 hours the formation of a new morphological r3/4 boundary was associated with expression of *Fgf-3* transcripts ($n=27$; data not shown).

By grafting rhombomere tissue to different axial levels, Guthrie and Lumsden (1991) demonstrated that new morphological boundaries were generated whenever even-numbered tissue was juxtaposed to odd but not when like confronted like. These data suggested that the formation of boundaries may be associated with a cell surface property of rhombomere cells which varies with a two segment periodicity. In the hindbrains of embryos older than stage 13, *Fgf-3* expression distinguishes a population of cells within rhombomere boundaries. Based on the studies of Guthrie and Lumsden, it might be predicted that *Fgf-3* transcripts would only be induced in similar grafting experiments at interface regions where odd-numbered tissue is confronted by even but not where like confronts like. We have investigated this proposition using the experimental regime of Guthrie and Lumsden (1991); in these experiments only tissue from within the body of the rhombomeres were confronted to exclude the possibility that existing boundary cells would be introduced into the interface region.

As expected, when r3 was confronted with r4, a new morphological boundary formed. Examination of *Fgf-3* expression in such experiments showed that transcripts had been induced in the new boundary ($n=10$; Fig. 3E,G). By contrast, when r3 was confronted with r5, no morphological boundary developed and no *Fgf-3* expression was observed in the interface region between host r3 tissue and donor r5 tissue ($n=10$; Fig. 3F, H). *Fgf-3* expression was also detected in unoperated boundaries. However there was a noticeable reduction in apparent *Fgf-3* RNA levels in all boundaries in these experiments; presumably a consequence of the experimental procedures.

Expression in the midbrain/hindbrain junction

We examined the expression of *Fgf-3* in other regions of the neuroepithelium and, interestingly, detected transcripts at the boundary between the midbrain and hindbrain (the isthmus), although not at the interfaces of more rostral neuromeres. The onset of detectable *Fgf-3* expression in the midbrain/hindbrain junction occurred at stage 11 and was maintained thereafter (13 somites, Fig. 2M–O). Transcripts were observed throughout the dorsoventral axis in this region and were detected just prior to the time when the neuroepithelium is committed to a cerebellar fate (Alvarado-Mallart, 1993). The cerebellar primordium extends either side of the midbrain/hindbrain junction (Martinez and Alvarado-Mallart, 1989) and *Fgf-3* transcripts encompass at least part of this region. Both the apparent level and complexity of *Fgf-3* expression in this region increase during development; by stage 24 (Fig. 3D), RNA is detected in two distinct regions in the posterior midbrain and anterior

hindbrain. These patterns of expression were also observed in stage 25 embryos; the latest stage examined in this study.

Expression in the pharyngeal region

Fgf-3 transcripts have been demonstrated in the pharyngeal pouches of the mouse embryo commencing at late stages of neurulation (Wilkinson et al., 1988). In the chicken embryo, *Fgf-3* expression was detected in the developing pharyngeal endoderm as early as stage 8+ (5 somites, Fig. 2I) in a longitudinal column of cells located lateral to the developing hindbrain extending anteriorly from the first somite position to the mid-hindbrain level. Expression became more intense by stage 9+ (8 somites) At stage 10+ (11 somites, Fig. 2L) when the initial single column of *Fgf-3*-positive cells appeared to divide into two separate domains, transverse sections showed that this tissue was the endoderm of the first and second pharyngeal pouches (Fig. 2G). Initially, increased transcript levels were observed in the first pouch only (stage 11+, 14 somites; Fig. 2M), but later *Fgf-3* expression was elevated in the second pharyngeal pouch (Figs 2N, 3J). *Fgf-3* expression was also observed in third pharyngeal pouch as it developed (data not shown).

Fgf-3 expression was also detected in the ectoderm of the second pharyngeal arch at stage 11– (12 somites, data not shown) and this expression continued until approximately stage 12+ (17 somites, Fig. 2O), after which expression became associated with the ectoderm of the third pharyngeal arch (data not shown). Transcripts were never observed in ectoderm of the first pharyngeal arch. Also, at stage 15 (25 somites), *Fgf-3* RNA was detected in lateral ectoderm (stomodeum) of the developing oral cavity (Fig. 3J).

DISCUSSION

FGF-3 expression identifies a population of cells in the midline of the cranial neural plate

In the cranial region of pre-somitic embryos, *Fgf-3* transcripts are restricted to midline neural plate cells and are absent from the developing notochord. This region of chick neural plate cells has not been formally named in the chick embryo but in *Xenopus*, is described as the notoplate (Jacobson and Gordon, 1976; Keller et al., 1985). In amphibians it comprises a specialised group of ectodermal cells which converge to the midline and extend rostrally so increasing the length of the neural plate and eventually is the region from which the ventral part of the cranial neural tube including the floor plate is derived. In *Xenopus*, the notoplate may also possess neural inductive properties (Ruiz i Altaba, 1992): FGF-3 may contribute to this signal and/or may be involved in convergent-extension movements in avian embryos. Cells extend rostrally from Hensen's node to form the cranial region of the notochord and the overlying cells of the midline of the neural plate (Selleck and Stern, 1991); during this extension process *Fgf-3* expression provides a marker that distinguishes these two populations. *Hnf-3 β* in the mouse embryo (Saski and Hogan, 1993) and sonic hedgehog in chicken and mouse embryos (*shh*; Riddle et al., 1993; Echelard et al., 1993) are also expressed early in the midline of the cranial neural plate. Moreover, an interaction between *shh* and FGF-4 has been demonstrated during limb development (Niswander et al., 1994); this raises

the possibility that FGF-3 may be involved in the regulation of *shh* in the cranial midline. Thus, FGF-3 may be involved in the development of the floor plate region and in dorsoventral patterning of the chicken neural tube. However, although *Fgf-3* expression was detected in the cranial neural plate of the chicken embryo this has not been reported for the mouse embryo nor for the notoplate of the amphibian embryo. The loss of *Fgf-3* in the midline region between stages 6 and 7 coincides with the beginning of elevation of the neural folds in the chick embryo (Schoenwolf and Smith, 1990) which may also indicate the time of floor plate formation.

FGF-3 in the posterior hindbrain and its role as an otic inducer

Our data suggest that there are two independent aspects to *Fgf-3* regulation and function in the developing avian hindbrain: the first relating to the dynamic pattern of expression in r4, r5 and r6 during the period of hindbrain morphological segmentation and the second related to the later development of rhombomere boundaries. What are the possible role(s) of FGF-3 during the early stage of hindbrain development? The otic placode, which invaginates to form the otocyst from which the sensory structures of the inner ear are derived, develops adjacent to r5 and r6 in mouse and chicken embryos. There is a body of evidence which suggests that signals from the hindbrain neuroepithelium regulate placode formation and subsequent development (reviewed by Van der Water and Represa, 1991) and the expression of *Fgf-3* in r5 and r6 of the mouse hindbrain suggested roles in the regulation of otic development (Wilkinson et al., 1988). In the chicken embryo the otic placode appears at stage 9, invagination begins at stage 10 and is largely complete by stage 15 (Meier, 1978). There is some evidence that signals produced by the hindbrain neuroepithelium between stages 9 and 17 promote this invagination to form the otocyst (Alvarez and Navascues, 1990). Anti-sense oligonucleotides and FGF-3 antisera have been shown to inhibit otic pit invagination in stage 10 chicken embryo explant cultures, effects that can be rescued by exogenous FGF-2 (Represa et al., 1991). However, in the absence of sequence information from the chicken *Fgf-3* gene, the oligonucleotides used to inhibit invagination were designed to the amino-terminal region of the mouse gene and the antiserum to a peptide from the human gene. Our results suggest that *Fgf-3* RNA is initially expressed within r4 and r5 and, only later, in r6. At the onset of placodal invagination, *Fgf-3* transcripts are detected in r4 and only in the anterior of r5 and, thus, their presence does not correlate spatially with otic vesicle invagination. In addition, *Fgf-3* expression in the *Xenopus* hindbrain appears to be at a level rostral to the invaginating otocyst (Tannahill et al., 1992). Of the reagents used to inhibit otic vesicle invagination in embryonic explants, the peptide sequence against which the antiserum was raised is completely conserved (residues 117–134; no. OA-11-835 Cambridge Research Biochemicals, UK) in the predicted chicken protein sequence. However, only 12 of 15 residues are conserved in the antisense oligonucleotide. Our own preliminary studies suggest that injection of either an identical antisense oligonucleotide or the same antibody preparation into embryos in vivo does not affect otocyst invagination (I. M. and A. Lumsden unpublished observations). Moreover, FGF-3 null mice show apparently normal otocyst invagination (Mansour et al., 1993)

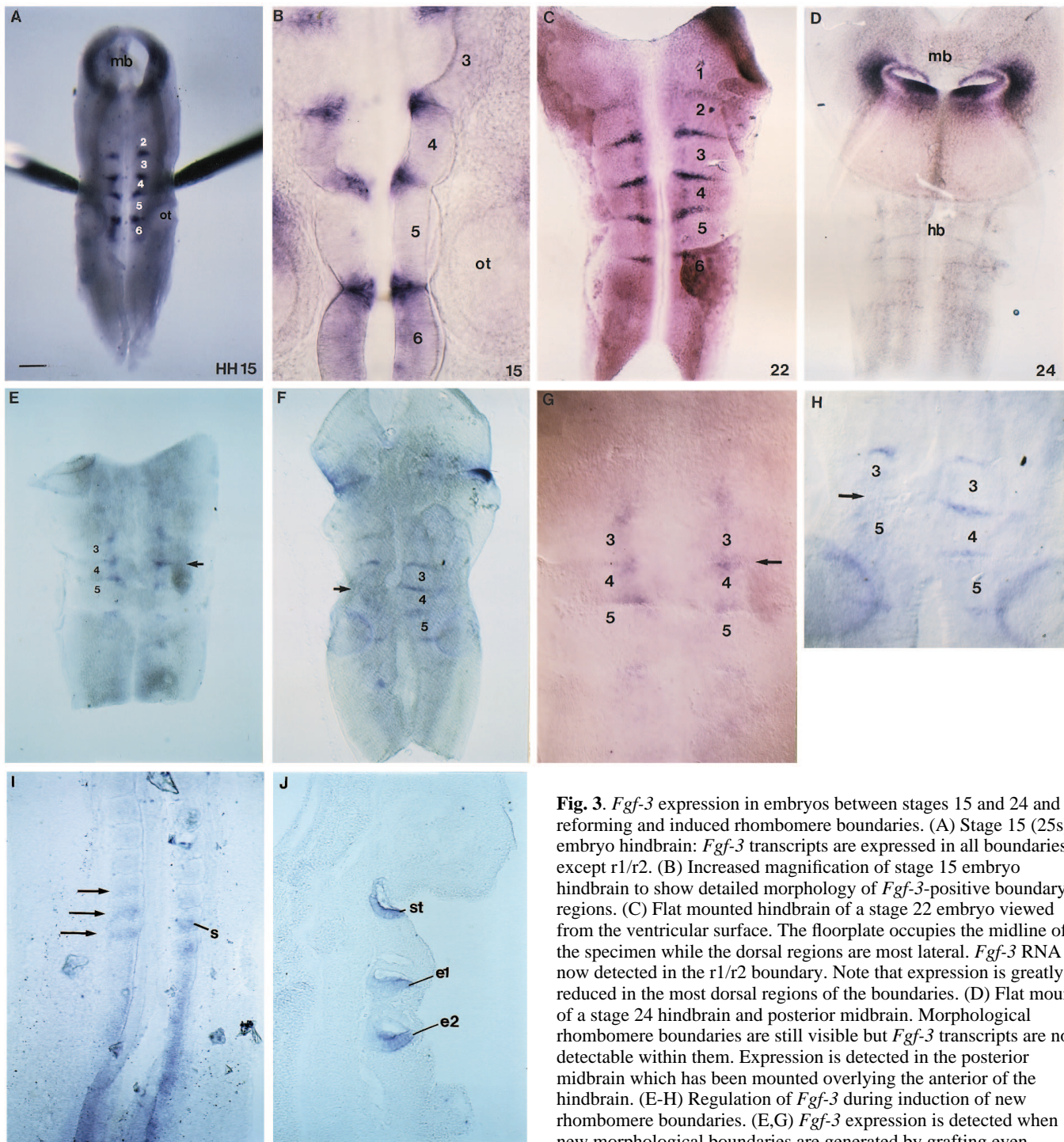


Fig. 3. *Fgf-3* expression in embryos between stages 15 and 24 and in reforming and induced rhombomere boundaries. (A) Stage 15 (25s) embryo hindbrain: *Fgf-3* transcripts are expressed in all boundaries except r1/r2. (B) Increased magnification of stage 15 embryo hindbrain to show detailed morphology of *Fgf-3*-positive boundary regions. (C) Flat mounted hindbrain of a stage 22 embryo viewed from the ventricular surface. The floorplate occupies the midline of the specimen while the dorsal regions are most lateral. *Fgf-3* RNA is now detected in the r1/r2 boundary. Note that expression is greatly reduced in the most dorsal regions of the boundaries. (D) Flat mount of a stage 24 hindbrain and posterior midbrain. Morphological rhombomere boundaries are still visible but *Fgf-3* transcripts are not detectable within them. Expression is detected in the posterior midbrain which has been mounted overlying the anterior of the hindbrain. (E-H) Regulation of *Fgf-3* during induction of new rhombomere boundaries. (E,G) *Fgf-3* expression is detected when new morphological boundaries are generated by grafting even-numbered rhombomere tissue adjacent to odd-numbered tissue. *Fgf-*

3 transcripts are shown in a morphological boundary (arrows) induced by grafting r4 tissue adjacent to r3. (F,H) *Fgf-3* expression is not induced when odd-numbered rhombomere tissue is grafted next to odd. *Fgf-3* RNA is not detected at the interface of r5 tissue grafted adjacent to r3. (I) Caudal region of stage 15 (25s) embryo showing *Fgf-3* transcripts in the segmental plate and the posterior half of the three most recently cleaved somites. (J) Sagittal section of a stage 15 embryo showing expression of *Fgf-3* in the stomodeum and endoderm of the first and second pharyngeal pouches. hb, hindbrain; mb, midbrain; ot, otocyst; s, somite; sp, segmental plate; st, stomodeum; e1 and e2, endoderm of the first and second pharyngeal pouches respectively. Rhombomeres are numbered. Scale bar in A represents 31 μ m (A), 5 μ m (B), 10 μ m (C,D,G-I) and 20 μ m (E,F).

and, although *Fgf-3* expression in r5 and r6 of the hindbrain of the *Kreisler* mutant mouse is abnormal, there is again no apparent defect in otocyst invagination (Frohman et al., 1993;

McKay et al., 1994). Together with our own data, these studies suggest that the role of FGF-3 in otocyst invagination may need to be re-evaluated.

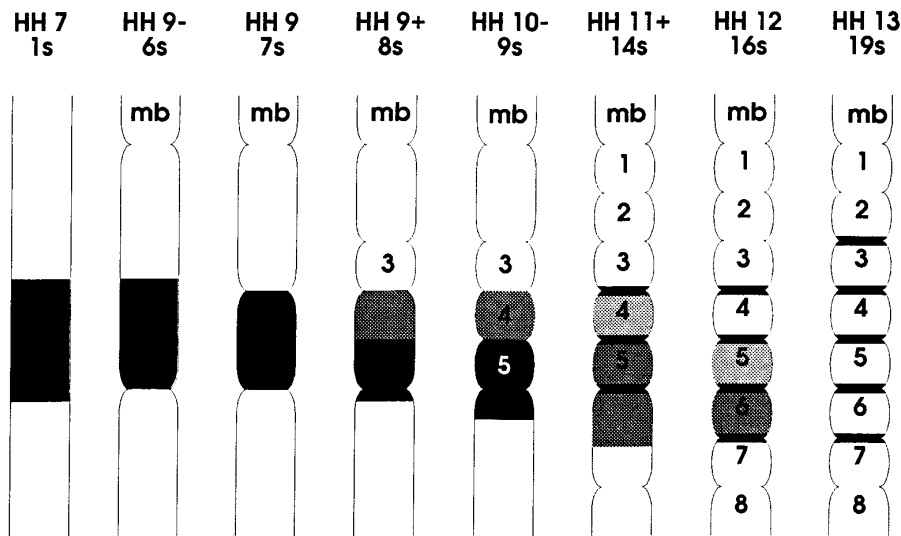


Fig. 4. Spatial and temporal regulation of *Fgf-3* RNA during chicken hindbrain development. *Fgf-3* expression (filled areas) is indicated together with the temporal appearance of morphological boundaries (adapted from Vaage, 1969 and Lumsden, 1990). *Fgf-3* RNA is present in posterior boundaries coincident with, or prior to, their formation but is only detected in the r1/2 and r2/3 boundary regions after a morphological boundary is present.

While otocyst formation is unaffected by targeted disruption of the murine *Fgf-3* gene, defects are observed during the subsequent development of this structure, in particular there are abnormalities in endolymphatic duct formation albeit with incomplete penetrance (Mansour et al., 1993). These may be related to *Fgf-3* expression in the hindbrain or to expression in the otocyst itself, which has been described in the mouse embryo (Wilkinson et al., 1989). In this regard it is important to note that we did not detect any expression in the otocyst of the chicken embryo at the stages analysed.

Local signalling events within the hindbrain neuroepithelium clearly play a major role in its development. This is best exemplified by observations that adjacent even-numbered rhombomeres regulate the production of neural crest cells by r3 and r5 by inducing *BMP-4* expression in them and exogenous *BMP-4* causes a massive reduction in neural crest production by these rhombomeres in vitro (Graham et al., 1993, 1994). *FGF-3* may also have functions in local signalling within the developing hindbrain. An important difference between the patterns of *Fgf-3* expression and those of other known segmentally restricted signalling molecules is that its domain of expression extends progressively more caudal during development and its posterior limit does not respect rhombomere boundaries at all times (see Fig. 4). This suggests that it may influence temporal aspects of rostrocaudal development in the posterior hindbrain.

The novel data produced in our studies in the chicken embryo have prompted us to re-examine expression in the developing hindbrain of the mouse. In this species we also detect a dynamic pattern of expression in r4, r5 and r6 followed by restriction to rhombomere boundaries, although expression in boundary regions is much weaker than observed in the chicken embryo (R. M., I. M. and G. Morriss-Kay unpublished data).

FGF-3 distinguishes rhombomere boundary cells and is regulated during boundary regeneration

Fgf-3 RNA expression provides an intracellular marker that distinguishes a population of cells in rhombomere boundaries during or soon after boundary formation; earlier than previously described molecular specialisations of boundaries

(Lumsden and Keynes, 1989). Individual rhombomeres are domains of lineage restriction following their morphological appearance, although some cells may be able to cross boundaries at later stages of hindbrain development (Fraser et al., 1990; Birgbauer and Fraser, 1994). Two mechanisms have been proposed to account for this restriction (Lumsden, 1990). The first is that adjacent rhombomeres differ in a cell surface property that renders their cells immiscible. This has received indirect support from studies of rhombomere boundary formation and direct support from studies of the dispersal of rhombomere cells in orthotopic and heterotopic grafts (Guthrie and Lumsden, 1991; Guthrie et al., 1993). Alternatively, rhombomere boundaries may contain a distinct population of cells which act as a mechanical barrier to prevent mixing of cells between adjacent rhombomeres. Consistent with this proposal, boundaries have a morphology that is distinct from the central regions of rhombomeres. The apical endfeet of cells within boundaries have a characteristic fan-shaped appearance and boundary cells make contacts with both the pial and ventricular surfaces (Heyman et al., 1993). There is also significantly increased extracellular space between boundary cells than in other regions of rhombomeres (Heyman et al., 1993), a corresponding reduction in gap-junctional contact (Martinez et al., 1992) and reduced rates of cell division associated with the accumulation of S-phase nuclei at the ventricular surface (Guthrie et al., 1991). Molecular markers that distinguish boundary regions at late stages of their development (stage 17) include the extracellular matrix glycoprotein laminin, the non-sialylated form of NCAM (Lumsden and Keynes, 1989) and a peanut lectin-binding epitope (Layer and Alber, 1990). However, these molecular characteristics may arise from the invasion of boundary regions by axonal processes, which may be defined by the presence of Ng-CAM, or endothelial cells at this time (Lumsden and Keynes, 1989; Noden, 1991).

We now show that *Fgf-3* RNA expression provides a marker that distinguishes a population of cells in rhombomere boundaries at earlier stages of hindbrain development providing further evidence for the existence of a specialised boundary cell population. However, it is possible that not all boundary cells express *Fgf-3* since RNA appears to be only weakly

detectable and in a much narrower strip of cells in the dorsal region of boundaries.

There is no simple relationship between the detection of *Fgf-3* transcripts and the appearance of morphological boundaries (Fig. 4). Expression of *Fgf-3* in presumptive boundary territories precedes morphological boundary formation in r3/r4, r4/r5, r5/6 and r6/r7, but appears only after boundary formation in the case of r1/r2 and r2/r3 boundaries. However, these data are further complicated by expression throughout the r4-r6 region at early stages of rhombomere formation and it seems likely that expression presages boundary formation due to overlap with this earlier pattern of expression. Interestingly, expression is never detected in the r7/r8 boundary raising the question of whether this represents a normal boundary region and it is notable that cell lineage restriction has not been demonstrated at this boundary. Experiments in which boundaries are formed and regenerated show that production of a new morphological boundary region is associated with induction of *Fgf-3* expression. Grafting of like-numbered rhombomere tissue adjacent to like fails to generate a morphological boundary and fails to induce *Fgf-3* expression. The use of *Fgf-3* as a marker in these studies provides further evidence that the structures formed in such experiments are indeed boundaries and for the involvement of a cell surface property with a two segment periodicity in their generation. It is also noteworthy that *Fgf-3* expression is induced in regeneration experiments within 8 hours of removal of the original boundary. Given that the neuroepithelium must first regenerate across the gap prior to boundary induction, a process that takes about 4 hours (Guthrie and Lumsden, 1991), it is likely that *Fgf-3* expression is regulated early during the reformation process.

What could be the function of *Fgf-3* expression in boundary regions? The appearance of transcripts only after the generation of the r1/2 and r2/3 boundaries suggests that FGF-3 is not involved in the process of cell lineage restriction. However, FGF-3 may regulate the development of later boundary specialisations including axon pathfinding and blood vessel formation. Rhombomere boundaries are preferred routes of axon projection by reticular neurons (Lumsden and Keynes, 1989) and there is a tendency for motor neurons arising close to boundaries in odd-numbered rhombomeres to extend axons within their adjacent boundary prior to crossing to their exit points in even-numbered rhombomeres (Guthrie and Lumsden, 1992). They are also the initial sites of blood vessel formation (Noden, 1991). FGFs are potent stimulators of neovascularisation and also promote neurite outgrowth in many populations of neurons in vitro, although neurotropic activity has not been demonstrated. This raises the possibility that FGF-3 might be involved in either process within boundary regions. Interestingly, the reticular neurons project ventrally towards the floor plate (Lumsden and Keynes, 1989) and this correlates with elevated expression of *Fgf-3* in the medial and ventral regions of boundaries but not the dorsal region. Thus FGF-3 may provide a directional cue for axonal projection within boundaries. Accumulation of axons is also observed in experimentally induced or reformed boundaries (Guthrie and Lumsden, 1991) correlating with earlier *Fgf-3* expression in such regions.

FGF-3 and mesoderm formation

Fgf-3 transcripts in the chicken embryo are first detected in the

primitive streak region where expression is observed along the length of the streak and in Hensen's node, with RNA detected in the epiblast, in the mesendoderm associated with the streak and in mesoderm lateral to the primitive streak. This is similar to the pattern of expression reported in *Xenopus* embryos in the blastopore region and maintained in the tail buds of older embryos (Tannahill et al., 1992). In the mouse embryo, by contrast, a similar pattern of *Fgf-3* RNA distribution is observed in mesoderm leaving the primitive streak but no transcripts are observed in the epiblast (Wilkinson et al., 1988; Niswander and Martin, 1992).

It has previously been proposed that FGF-3 might regulate the proliferation and/or migration of mesoderm in the region of the primitive streak (Wilkinson et al., 1988), however targeted disruption of the mouse *Fgf-3* gene does not result in defects consistent with this hypothesis (Mansour et al., 1993).

In amphibian embryos, FGFs have been implicated in the induction of ventral mesodermal derivatives (see Slack, 1994, for most recent review and references). However, it is unlikely that FGF-3 is involved in mesoderm induction in the avian embryo since expression in primitive streak epiblast is first detected after the onset of mesoderm formation (Bellairs, 1960). Likewise, the absence of maternal expression in *Xenopus* suggests that FGF-3 is unlikely to perform this function in amphibians (Tannahill et al., 1992) although it does have mesoderm-inducing capabilities in vitro (Paterno et al., 1989).

In older chick embryos, *Fgf-3* transcripts are detected in the segmental plate, the most recently formed region of the notochord, and in the posterior part of the three most recently formed somites. *Fgf-3* is similarly expressed in the mouse embryo (R. M., I. J. M. and G. Morriss-Kay unpublished data) and these observations may relate to the unexpected tail defects including disorganised somites and abnormal caudal vertebrae observed in FGF-3 null mice (Mansour et al., 1993).

Concluding remarks

Considering the complex and dynamic pattern of *Fgf-3* expression in both the chicken and mouse embryos, it is surprising that the FGF-3 null mutant mouse shows no apparent defects of gastrulation, pharyngeal arch formation and hindbrain development and differentiation (Mansour et al., 1993). However, subtle changes for example in the developing hindbrain might not be detected since its developmental neuronal anatomy is not well described. Moreover, it is noteworthy that while 25% of embryos in utero are homozygous for the null mutation only 11% of those born are homozygotes (Mansour et al., 1993), suggesting that there may be additional phenotypes in a subset of embryos. Further understanding of FGF-3 function in vertebrate embryos clearly awaits the identification of its receptor(s); a knowledge of receptor expression will facilitate the identification of potential target cell populations and the construction of more precise hypotheses concerning FGF-3 function.

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